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Apoptosis and Liver Disease

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Considering the important role of apoptosis in a growing number of physiological and pathophysiological conditions, it is interesting to note that research in this field is surprisingly young. Although the term apoptosis was first introduced by Kerr in 1972 (1), little was known about apoptosis until the mid-1980s. However, in the last 15 years things have changed considerably, and by now papers published on apoptosis are growing exponentially each year. Especially in the past few years, significant advances have been made in our understanding of cell death by apoptosis. Indeed, apoptosis has now emerged as a fundamental process in tissue homeostasis and is vital for the necessary balance between cell loss and cell gain in normal tissue (2). In normal tissue, rates of mitosis are therefore counterbalanced by rates of apoptosis (3). Of equal importance, apoptosis is nature's way of eliminating unwanted, senescent, and damaged cells from multicellular organisms (4). Given the pivotal role of apoptosis in cell homeostasis, it is not surprising that basic apoptotic mechanisms are highly conserved in evolution (5).

Any kind of dysregulation of apoptosis is potentially deleterious and can have profound consequences. The liver is no exception, and we now realize that dysregulation of apoptosis is a principal mechanism contributing to many liver diseases. Indeed, excessive apoptosis can lead to severe liver damage, as can be exemplified by fulminant hepatic failure seen after experimental induction of apoptosis in mice (6). On the other hand, failure of apoptosis has been implied as a major determinant in development of hepatocellular carcinoma such as occurs with mutations of p53 (7). Treatment strategies to moderate apoptosis are therefore desirable to inhibit apoptosis in liver injury and selectively induce apoptosis in malignant liver tumors. Apoptosis is not only important in the pathophysiology of human liver diseases, it is also on the edge of entering clinical practice by providing new treatment opportunities. Our intention is to provide a useful overview of the current knowledge of apoptosis in liver diseases, especially for the reader new to the field of apoptosis. Although apoptosis has been identified in a variety of human liver diseases (8), in this review we will focus on alcoholic liver disease, viral hepatitis, cholestatic liver diseases, and hepatocellular carcinoma (HCC).

DEFINITION AND IDENTIFICATION OF APOPTOSIS

Apoptosis is a form of cell death characterized by organized nuclear and cellular fragmentation. During apoptosis, cells are fragmented into small membrane-bound bodies with intact organelles and plasma membrane. Ordered DNA fragmentation is a biochemical characteristic of apoptosis. Endonucleases first cleave the DNA into large 50- to 300-kb pieces and then further into smaller 180 to 200 base pair fragments, which are responsible for the classic ladder pattern found in most apoptotic cells (9). Ultimately, the apoptotic cells, then called apoptotic bodies, are removed by phagocytosis in vivo (10). In the liver, apoptotic bodies have long been recognized in histopathologic specimens and have been referred to as Councilman bodies. The whole process is fast, ranging from minutes to a few hours (11). Normally, apoptotic cells are eliminated without an inflammatory response, because intracellular constituents are contained in the membrane-bound apoptotic bodies. Although this seems to be true in general, apoptosis in the liver is less silent than previously thought, as can be exemplified by the detection of intracellular enzymes during hepatocyte apoptosis. Furthermore, if the magnitude of apoptosis is sufficient to overwhelm the removal process, loss of tissue architecture occurs with an inflammatory response (12). Thus, apoptosis may play a key role in many diseases thought to be initiated in the past by necrosis.

Because apoptosis is a fast process, a combined biochemical and morphologic approach is usually used to identify apoptotic cells before they are cleared by phagocytosis. Annexin proteins specifically bind phosphatidylserine, which is normally confined to the cytoplasmic leaflet of cellular membranes. Early during apoptosis, this phospholipid asymmetry is lost and phosphatidylserine is

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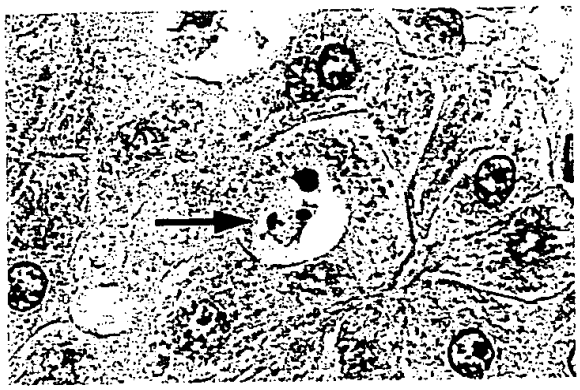

Figure 1. Example of hepatocyte apoptosis in a patient with cholestasis after liver transplantation.

translocated to the outer part of the plasma membrane, where it is accessible to labeled annexin V (13). Fluorescently labeled annexin V is frequently used to identify apoptotic cells in experimental conditions.

Different methods are also used to detect the characteristic DNA fragmentation of apoptosis. One widely used example is the TUNEL technique (terminal deoxynucleotide transferase–mediated deoxyuridine triphosphate nick end-labeling), which labels the 3′-OH ends of cleaved DNA fragments (14). Hematoxylin- and eosin-stained liver tissues can be screened for apoptosis using light microscopy. Apoptotic hepatocytes will show as round acidophilic bodies that are detached from the surrounding cells and may have a pyknotic or fragmented nucleus (Figure 1). As mentioned above, these acidophilic bodies are the classic Councilman bodies seen in a variety of human liver diseases (15). The characteristic ultrastructural features of apoptosis, such as cytoplasmic blebs and chromatin condensation, also can be readily identified in tissues or single cells by transmission electron microscopy (16). If such biochemical methods as the TUNEL assay are correlated with typical morphologic evidence, an accurate assessment of apoptosis can be achieved in most models and liver diseases. In the future, the identification of apoptosis may be made by demonstrating activation of apoptosis effector molecules by immunohistochemistry. For example, immunohistochemistry for neoepitopes of caspases (see below) may become a preferred method for identifying apopotic cells.

MECHANISMS OF APOPTOSIS

During apoptosis, a final or execution phase can be distinguished from an initiation phase. In the execution phase, caspases, a new family of proteases, dismantle the cell by sequential activation and cleavage of key proteins (17). Caspases are present in the cytosol of most cells as zymogens and need to be activated to fully functional proteases by cleavage of the proenzyme by proteolytic steps. Caspases cleave proteins on the carboxyl side of aspartate moieties. Because caspases themselves are activated by cleavage of aspartate residues, it is generally thought that caspases are activated by other caspases. Caspase activation by other caspases may result in a caspase cascade analogous to the coagulation cascade. Certain caspases (−3, −6, −7) are called effector or downstream caspases, because they cleave key substrates and eventually lead to apoptotic cell death. Currently, two major pathways are known to activate these effector caspases. One involves death factors and death receptors, the other mitochondrial dysfunction (18).

Death receptors are members of the tumor necrosis factor (TNF) superfamily. To date, eight death receptors have been described, of which TNF receptor-1 (TNF-R1) and Fas (CD95/APO-1) are best characterized. Upon activation by their ligands, both Fas and TNF-R1 recruit a discrete intracellular death complex consisting of adapter proteins and procaspases. The death complex then activates a class of apical caspases, most notably caspase-8, which subsequently activates the downstream effector caspases (19).

In the second pathway, cellular stress can trigger release of cytochrome c from mitochondria, which is probably mediated by permeability changes of the inner mitochondrial membrane, called the mitochondrial permeability transition (MPT) (20). Cytochrome c then binds to Apaf-1, which in turn activates caspase-9. From here on the pathways converge, because caspase-9 activates the downstream caspases mentioned earlier (21). Recent data suggest that the mitochondrial and death receptor pathway are connected by the protein Bid, which can induce cytochrome c release from mitochondria after cleavage and activation by caspase-8 (22). The different pathways leading to apoptosis are summarized in Figure 2.

Several cytoplasmic proteins are critically involved in the regulation of apoptosis, in particular members of the Bcl-2 family. At least 15 different Bcl-2 proteins have been identified and can be subdivided into pro- and anti-apoptotic members (23). Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bfl-1, Brag-1, and A1 inhibit apoptosis, whereas Bax, Bak, Bcl-xS, Bag, Bid, Bik, Hrk, and Bad promote apoptosis in mammalians. Pro- and anti-apoptotic Bcl-2 members can associate and form complexes, thereby titrating their respective function. Apparently, anti-apoptotic Bcl-2 family proteins can also bind to mitochondria and inhibit the release of cytochrome c (24). Although the precise functions of Bcl-2 family members in the modulation of apoptosis are still unclear, the balance between the various Bcl-2 family members in a cell will finally determine whether an apoptotic stimulus can cause the cell to undergo apoptosis.

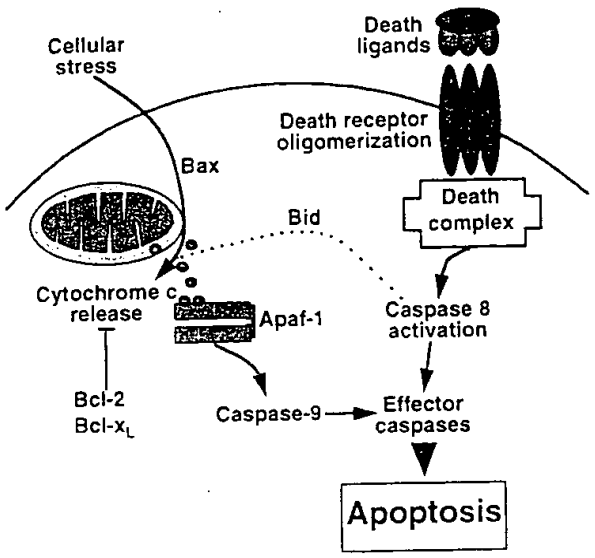

Figure 2. Intracellular signaling pathways for apoptosis. Two pathways can activate the effector caspases, which lead to apoptotic cell death. In the first one, death ligands activate their receptors, resulting in the formation of a death complex, which then activates caspase-8. In the second pathway, cellular stress leads to mitochondrial dysfunction with release of cytochrome C into the cytoplasm. Cytochrome C then binds to Apaf-1 and activates it, followed by activation of caspase-9. A link between both pathways is the protein Bid, which can be activated by caspase-8 and leads to a release of cytochrome C from mitochondria. Cytochrome C release can be blocked by anti-apoptotic Bcl-2 family members and promoted by pro-apoptotic members of this family, such as Bax.

APOPTOSIS AS A MECHANISM OF LIVER DISEASE

Alcoholic Liver Disease

To date, only limited information is available on the pathophysiology of apoptosis in alcoholic liver disease. Yet it has become evident that apoptosis is important in both clinical and experimental alcohol-induced liver diseases. Indeed, apoptosis is represented in human alcoholic liver disease in the form of acidophilic bodies. Furthermore, markers of apoptosis and Mallory bodies can be seen in hepatocytes at the same time, indicating that those cells might be removed from the tissue by apoptosis (25,26). An increasing number of studies also show that apoptosis occurs in experimental alcoholic liver disease. Chronic alcohol administration in mice resulted in a significant increase of apoptotic bodies in hepatocytes, especially around terminal hepatic venules. These changes were time dependent and reversible after discontinuation of alcohol (27). Another study demonstrated an increase of apoptotic cells in alcohol-fed rats with concomitant liver injury, such as fatty liver or liver inflammation (28). An underlying liver pathology might therefore make hepatocytes more susceptible to alcohol-induced apoptosis. This also seems to hold true for hepatic iron overload, which can be found in many cases of human alcoholic liver disease. Indeed, in a rat model used to study the effects of iron in the liver, a substantial increase of apoptotic cells was seen in those animals that had excess hepatic iron accumulation (29).

Several mechanisms seem to be involved in alcohol-induced apoptosis of hepatocytes. Alcohol-induced liver injury has been linked to oxidative stress caused by the production of reactive oxygen intermediates. Indeed, more apoptotic cells could be seen in rats with acute alcohol intoxication after glutathione depletion. Consistent with a role for oxidative stress in alcohol-mediated liver injury, antioxidants could reduce the rate of apoptosis in the same model (30). One important mechanism for the formation of reactive oxygen intermediates and lipid peroxides appears to be the induction of cytochrome P450 2E1 (CYP2E1), which is highly expressed in the liver (31). In a human hepatocyte cell line, which has been transduced to express CYP2E1, enhanced lipid peroxidation by reactive oxygen intermediates and subsequent apoptosis can be seen when these cells are enriched with arachidonic acid, a polyunsaturated fatty acid (32). The same cells could be protected against CYP2E1-induced apoptosis by transfection with Bcl-2. Interestingly, another study demonstrated an association of increased Bcl-2 protein concentration with markers of inflammation and lipid peroxidation in rats with experimental alcoholic liver disease (28). By expressing the anti-apoptotic protein Bcl-2, hepatocytes might therefore have a defense mechanism against alcohol-induced liver toxicity.

Oxidative stress has also been linked to the Fas-system. In patients with alcoholic liver damage, high Fas ligand mRNA expression was found in hepatocytes (33). This de novo Fas ligand expression appears to be induced by reactive oxygen intermediates (34). Because hepatocytes also express the Fas receptor, these findings suggest that hepatocytes might mediate their own death by a paracrine or autocrine mechanism (35). This potentially important mechanism of alcohol-induced liver injury has been termed fractricide (Figure 3).

Several other factors seem also to be involved in alcohol-related apoptosis. The fibrogenic cytokine transforming growth factor (TGF)–beta 1 is able to induce apoptosis in cultured hepatocytes (36). TGF–beta 1 is produced by hepatic stellate cells in alcoholic liver disease and might therefore have a double impact on the progression of disease: promoting fibrogenesis and killing hepatocytes by apoptosis. Finally, increased TNF–alpha 1 activity has been demonstrated clinically in patients with alcoholic liver disease (37) and experimentally in ethanol-fed rats with concomitant liver injury (38). Chronic ethanol administration also increases TNF–alpha 1 re-

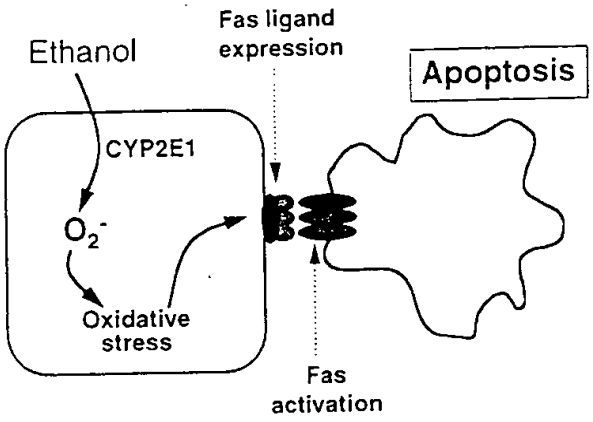

Figure 3. Ethanol induces fratricide by potential oxidative stress–induced Fas ligand expression. In hepatocytes exposed to ethanol, reactive oxygen intermediates are produced by induction of cytochrome P450 2E1 (CYP2E1). This oxidative stress then leads to induction of Fas ligand expression and paracrine killing of Fas-receptor-positive hepatocytes (fractricide).

ceptors on hepatocytes (39,40). Taken together, the up-regulation of the TNF–alpha 1 system during ethanol exposure renders hepatocytes very susceptible to apoptosis caused by this cytokine, suggesting an important role of TNF–alpha 1 in the pathophysiology of alcoholic liver disease.

Viral Hepatitis

There is accumulating evidence that apoptosis of liver cells plays a significant role in the pathogenesis of viral hepatitis. Pathomorphologic studies have shown that apoptotic cell death is present in human hepatitis. Indeed, hepatocyte dropout as a result of apoptosis is frequently observed (41). As mentioned earlier, acidophilic or Councilman bodies have also been recognized as an expression of apoptosis in liver disease. The apoptosis associated with viral infections is generally thought to be effected by cytotoxic T lymphocytes (CTL). Different apoptotic pathways have been implicated in this process including the Fas and TNF-alpha system (42,43) as well as the perforin/granzyme system (44,45) (Figure 4).

There is only limited information on the role of apoptosis in acute hepatitis. A small study identified enhanced Fas protein expression by hepatocytes in three patients with fulminant hepatic failure resulting from hepatitis B virus infection. Most viable hepatocytes were also positive in the TUNEL assay, indicating an involvement of Fas-mediated apoptosis in the pathophysiology of acute hepatitis (46).

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the major causative agents of chronic liver disease. Viruses appear to sensitize hepatocytes to apoptosis and also to inhibit apoptosis depending on the model and viral protein studied (47). For example, in a rat hepato-

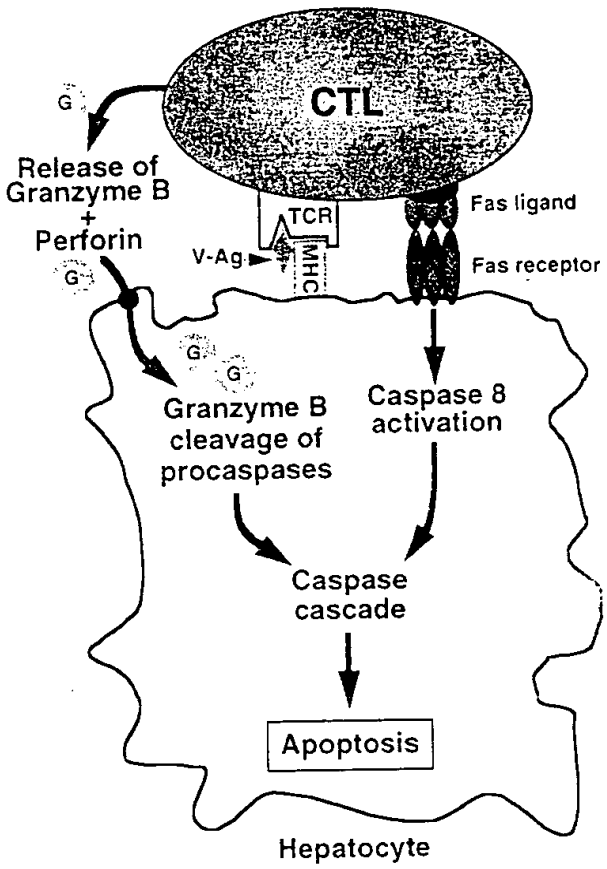

Figure 4. Cytotoxic T lymphocyte (CTL)–mediated apoptosis of virus-infected hepatocytes. Activated CTLs recognize viral proteins by the T-cell receptor in the context of mixed histocompatibility antigens. Binding of Fas ligand to Fas receptors on the virus-infected hepatocytes activates caspase 8 and triggers the caspase cascade, leading to apoptosis. In addition, CTLs also release cytotoxic granules containing granzyme B and perforin after engagement of the T-cell receptor. Internalization of granzyme B by the hepatocyte is mediated by formation of perforin-induced membrane pores. Granzyme B then cleaves procaspases that also lead to apopotosis (G = granzyme B; CTL = cytotoxic T lymphocyte; TCR = T-cell receptor; MHC = major histocompatibility complex; V-Ag = viral antigen).

cyte cell line, TNF-alpha induced extensive apoptosis only in those cells expressing a high level of HBV (48). The sensitization of liver cells to apoptotic stimuli during HBV infection has been linked to the X-gene product of HBV (49). Similarly, in HCV-infected cells, the viral core protein of HCV can bind to the cytoplasmic domain of TNF-R1. This interaction promotes cell death by apoptosis by means of TNF signaling pathways in mouse and human cell lines (50). The mechanism of the sensitization is still unknown but seems to be independent of an up-regulation of the TNF-R1. In order to complete their replication cycle and prevent clearance of infected cells, viruses have developed mechanisms to block apoptosis.

This can be exemplified by the NS3 protein of HCV, which can suppress actinomycin-induced apoptosis in an experimental setting (51). The overall effect of viral proteins on apoptosis likely will depend on the apoptotic stimulus, the cellular context, and the relative expression levels of different viral proteins.

There are also an increasing number of clinical studies showing that the immune-mediated apoptotic pathways are activated in chronic HBV and HCV infection. Expression of Fas and HCV core antigen was studied immunohistochemically in liver tissue of 40 patients with chronic HCV infection. The prevalence of Fas in HCV core antigen positive hepatocytes was significantly higher than in uninfected cells (52). An activated Fas system is also observed in livers from patients with chronic HBV infection, where Fas is not only highly expressed in hepatocytes but is also upregulated on a molecular level, as demonstrated by an increase of Fas mRNA (33). Interestingly, the Fas mRNA was found in areas with lymphocytic infiltration, thereby supporting the mechanism of CTL-mediated apoptosis of infected hepatocytes by the Fas system.

Cholestatic Liver Diseases

Cholestasis is observed in a variety of clinical syndromes and may be the main feature of a number of chronic progressive liver diseases, which finally lead to cirrhosis, liver failure, and death. Hepatic retention of hydrophobic, toxic bile salts has long been implicated as a major cause of liver damage (53). Accumulation of these toxic bile salts within the hepatocyte is thought to play a key role in liver injury during cholestasis. Indeed, hepatic levels of the toxic bile salts chenodeoxycholate and deoxycholate correlate with the degree of liver damage (54). Because widespread necrosis is not prominent in most cholestatic liver diseases, it became apparent that hepatocyte cell death during cholestasis occurs by apoptosis rather than by necrosis (41). For example, in liver tissue from patients with primary biliary cirrhosis (PBC), apoptotic features occur more frequently than in normal controls (55). The mechanisms of apoptosis by toxic bile salts have been partially elucidated in recent years. Toxic bile salts, such as glycodeoxycholate (GCDC), can directly cause apoptosis in cultured rodent hepatocytes (56). GCDC-induced apoptosis is initiated by a ligand-independent activation of Fas by this bile salt (57). Subsequently, caspase 8 is cleaved and eventually activates downstream caspases, leading to apoptosis. Cathepsin B, a cystine protease, is also important in this model of apoptosis. Indeed, cathepsin B is activated and translocated to the nucleus and participates as an effector mechanism in bile-salt–induced apoptosis (58). GCDC-induced apoptosis also appears to require an activation and translocation of protein kinase C (PKC), which then leads to an increase of intracellular magnesium (56,59). This causes

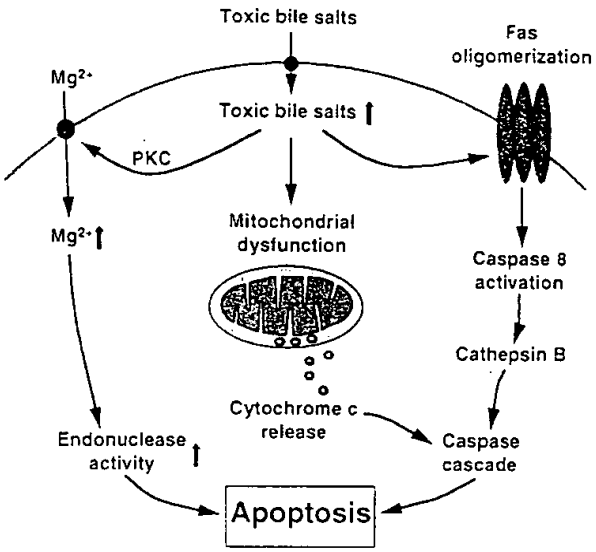

Figure 5. Mechanisms of bile salt–induced apoptosis in hepatocytes. Toxic bile salts can induce Fas oligomerization followed by activation of caspase 8, cathepsin B, and the capase cascade that finally leads to apoptosis. Toxic bile salts can also cause apoptosis by disrupting mitochondrial function with release of cytochrome C, which again activates the caspase cascade. In addition, endonucleases can be activated by a PKC-mediated increase of intracellular Mg2+ (see text for details).

an activation of Mg2+-dependent endonucleases, leading to DNA cleavage. The mechanisms of bile salt–induced apoptosis are summarized in Figure 5. Interestingly, coadministration of ursodeoxycholate (UDC), a nontoxic hydrophilic bile salt, with a toxic bile salt was shown to reduce apoptosis in a human hepatocyte cell line (60). The protective effect of UDC seems to be associated with a reduction of the MPT. Thus, reduction of apoptosis is likely to be one important mechanism for the beneficial effect of UDC in cholestatic diseases.

The role of Bcl-2 in bile-salt–induced apoptosis in hepatocytes has been investigated using the bile duct ligated rat as a model of cholestasis. Bcl-2 is not expressed in hepatocytes under normal conditions. However, de novo expression of Bcl-2 was observed in hepatocytes of bile duct–ligated rats, suggesting an adaptive mechanism to resist apoptosis by toxic bile salts (61). This is further supported by the observation that cholangiocytes, which are in direct contact with bile continuously, express Bcl-2 constitutively (62). De novo Bcl-2 expression was also found in liver tissue of patients with PBC (55), where it presumably functions in an anti-apoptotic role to prevent bile salt–mediated liver damage.

Hepatocellular Carcinoma

All the liver diseases mentioned before had one thing in common: a higher rate of apoptosis compared with healthy controls, leading to liver injury. In contrast, in-

sufficient apoptosis of DNA-damaged and malignant cells has been recognized as the major determinant in hepatocarcinogenesis. The available evidence indeed demonstrates a disruption of apoptosis in several steps of HCC development. An analysis of 22 HCCs revealed a partial or complete loss of Fas, which is normally expressed constitutively in hepatocytes (63). As shown by another study, Fas expression is correlated to the degree of HCC differentiation: especially in HCCs with very poor differentiation, portal tumor thrombus or extracapsular invasion, Fas expression was significantly reduced (64). These observations suggest that loss of Fas expression is one way to escape CTL cytotoxicity and thus promotes survival of malignant cells. On the other hand, a p53-dependent upregulation of Fas expression with subsequent apoptosis is seen in human hepatoma cells after treatment with different chemotherapeutic agents (65).

Dysregulation of apoptosis mediated by TGF-beta 1 has been implicated as an additional important factor in hepatocarcinogenesis. As mentioned before, TGF-beta 1 is able to induce apoptosis in normal hepatocytes (36). Signaling of TGF-beta 1 involves two distinct receptors, type 1 and type 2. TGFβ 1 binds selectively to the type 2 receptor, which then forms a complex with the type 1 receptor, leading to the generation of downstream signals (66). In HCC, a significant reduction in the mRNA levels for both receptors is observed and leads to an impairment of apoptotic signaling (67). In addition, another study suggested that although the type 2 receptor was expressed in tumoral hepatocytes, it was no longer detected on the plasma membrane but was intracytoplasmic in a diffuse pattern (68). This study suggests that escape from the proliferation control of TGF-beta 1 might be related to a defect in type 2 receptor processing on the liver cell membrane.

Finally, there is a positive correlation between the apoptotic rate in HCC and p53 protein expression, supporting the role of p53 in regulating apoptosis in neoplastic liver lesions (69). The protein p53 is a tumor suppressor gene necessary for cells to complete repair of DNA damage. If DNA repair is impossible, p53 can induce apoptosis. Thus, a dysfunctional p53 allows cells to escape apoptosis and leads to cancer development. Indeed, mutations of p53 with loss of its function have frequently been found in HCC (70,71).

SUMMARY

Based on the available information, apoptosis is clearly playing an important role in the pathophysiology of human liver diseases. An increased rate of apoptosis involving many different mediators, such as Fas, TNF-alpha, TGF-beta 1, and members of the Bcl-2 family is contributing to the liver injury seen in a variety of diseases, including viral hepatitis, cholestatic disorders, and alcoholic liver disease. A reduction of apoptosis should therefore be beneficial in these diseases and the goal for future drug development. On the other hand, dysregulation of apoptotic processes protects malignant hepatocytes from cellular suicide. Specific induction of apoptosis might therefore be a new option for the prevention and treatment of HCC.

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